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(54) Title: METHOD FOR PRODUCING TRANSGENIC ANIMAL MODELS WITH MODULATED PHENOTYPE AND ANIMALS PRODUCED THEREFROM

(57) Abstract

A method of preparing a transgenic animal model with enhanced, accelerated pathology for Alzheimer's Disease (AD) and the transgenic animal made by the method are disclosed. The method includes producing an F_1 generation by crossing a first and second transgenic parent each carrying a different expressible transgene for differing aspects of the same desired phenotype associated with AD pathology. The offsprings of the F_1 generation are then screened and those which carry a transgene from each parental transgenic animal resulting in an enhanced pathology for Alzheimer's Disease are selected. In a preferred embodiment the AD-associated pathology is for amyloid accumulation. In an embodiment a mutant presenilin transgene and a transgene for a mutant amyloid precursor protein are used. In a further embodiment the mutant presenilin transgene is the PS1 M146L mutation and the mutant amyloid precursor protein transgene is the Swedish mutation (app695 isoform containing a K670N, M671L mutation (app770 numbering)).

METHOD FOR PRODUCING TRANSGENIC ANIMAL MODELS
WITH MODULATED PHENOTYPE AND ANIMALS PRODUCED THEREFROM

FIELD OF THE INVENTION

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The present invention relates to the
production and use of transgenic animal models
including methods of modulating the phenotype resulting
from expression of the integrated transgenes. More

10　particularly, the invention relates to improving models
of neurodegerative disorders including models for
Alzheimer's Disease and the animals produced therefrom.

BACKGROUND OF THE INVENTION

15

Transgenic animals are being utilized as model
systems for studying both normal and disease processes.
In general an exogenous gene with or without a mutation
is transferred to the animal host system and the
20　phenotype resulting from the transferred gene is
observed. Other genetic manipulations can be
undertaken in the vector or host system to improve the
gene expression leading to the observed phenotype
(phenotypic expression). The gene may be transferred
25　under the control of different inducible or constituent
promoters, may be overexpressed, the endogenous
homologous gene may be rendered unexpressible and the
like. [PCT Application WO 92/11358; US patent
5,221,778] However, even with these manipulations the
30　desired phenotype is not always expressed (for example,
see herein below regarding PS transgenes). Further, as
set forth in US Patents 5,602,299 and 5,221,778 various
breeding programs to change the background, insert
additional transactivator transgene, make the transgene
35　homozygous or have hosts carrying two copies of the
transgene but inserted at two different sites have been

disclosed. However, again these methods do not always
improve the phenotypic expression of the transgene such
that the model system more closely resembles the
desired phenotype. Therefore additional methods are
5   needed to improve and/or modulate the phenotype of
transgenic animals.

Transgenic model systems are needed to study
neurodegenerative disorders, both to understand the
underlying disease pathology as well as to test
10   treatment protocols. Alzheimer's disease (AD) is a
neurodegenerative disorder with a progressive dementia
characterized by the presence of extracellular amyloid
deposits (composed mainly of β-amyloid (Aβ)) and
intraneuronal tangles, (consisting largely of the
15   cytoskeletal protein tau), in specific brain regions.
Its symptoms include gradual memory loss, declined
ability to perform routine tasks such as eating,
confusion, disorientation, the inability of the patient
to care for him or herself, and eventually death. The
20   American Health Assistance Foundation has reported that
presently more than four million Americans are believed
to have AD, and each year 100,000 Americans die because
of AD and 250,000 new cases of AD are diagnosed.
Moreover, one out of every ten Americans 65 years and
25   older have AD and almost half of those 85 years and
older have the disease.

Although AD in general is associated with patients
in their late 60s, 70s and older, Familial Alzheimer's
Disease (FAD) has been documented in patients in their
30   thirties and forties. FAD is genetic autosomal
dominant form of AD. Although the genetic causes of
FAD are not thought to be the same as AD, the FAD
phenotype appears to be pathologically similar to AD.
It has been reported that 10% of all AD cases are FAD.
35   Autopsies of patients who suffered from FAD have
shown the presence of neuritic plaques made up largely

of beta-amyloid (Aβ) and neurofibrillary tangles
consisting largely of deposits of tau protein [reviewed
in Hardy and Duff, 1993]. Beta-amyloid is a 40-42
amino acid peptide produced by the proteolytic cleavage
5   of the larger amyloid precursor protein (APP). APP is
a transmembrane protein with a single transmembrane
domain running from residue 700 to residue 722. The
APP gene is located on chromosome 21 and contains 18
exons. APP isoforms resulting from alternative
10 splicing form a set of polypeptides ranging from 563 to
770 residues in length. The beta amyloid fragment is
encoded by the 3' half of exon 16 and the 5' half of
exon 17, which also encodes APP's transmembrane domain.
Most of the beta-amyloid cleaved from APP is forty (40)
15 amino acid residues long and designated Aβ1-40.

Another form of beta-amyloid produced in much
smaller amounts relative to the production of Aβ1-40 is
a peptide 42-43 amino acid residues long. It is
designated Aβ1-42(43). This peptide is selectively
20 deposited early in the FAD process. Experiments
conducted in vitro have demonstrated this peptide forms
insoluble aggregates much faster than Aβ1-40. Hence it
is believed that increased production of Aβ1-42(43)
occurs in patients genetically predisposed to FAD and
25 initiates its pathology. Since both forms of beta-
amyloid are insoluble, they deposit on neurons in the
brain.

Genetic causes of AD include mutations in the APP
gene on chromosome 21, the presenilin 1 (PS1) gene on
30 chromosome 14 and the presenilin 2 (PS2) gene on
chromosome 1 [Goate et al, 1991; Sherrington et al,
1995; Levy-Lahad et al, 1995]. All known mutations
which cause AD have been shown to alter the processing
of APP such that more amyloidogenic Aβ (Aβ42(43)) is
35 generated. This had led to the hypothesis that
aberrant APP processing and the generation of Aβ42(43)

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may underlie the early etiopathogenesis of FAD
[Younkin, 1995; Scheuner, 1996; Cai et al, 1993; Citron
et al, 1992; Suzuki et al, 1994].

5   In some patients suffering from FAD, a missense
point mutation in exon 17 at a codon 717 (transcript
770) of the APP gene was determined responsible for the
increased formation of beta-amyloid, and hence FAD [PCT
Application PCT/GB92/00123]. Soon thereafter, other
point mutations were found in the same codon in

10   patients suffering from FAD [Hardy, 1993].

In an effort to develop animal models to study
pharmaceutical agents designed to treat FAD, transgenic
mice have been developed containing the missense point
mutations of the human APP gene in their genome [Hsiao

15   et al., 1996; Games et al., 1995; United States Patent
5,612,486; PCT Applications WO 92/06187; WO 93/14200;
WO 96/06927]. Since a mutated APP gene is expressed in
their brains, these transgenic mice have the potential
to serve as models for FAD. Models with overexpression

20   of the APP gene (with and without mutations) have also
been developed [PCT Application WO 94/24266; WO
96/06927; European Patent Application EP0 653 154 A2].

Some of these APP gene transgenic mice have been
shown to produce pathology which resembles that of FAD

25   at one year of age and older [Hsiao et al., 1996 and
Games et al., 1995]. The PDAPP mouse [Games, et al,
1995] expresses an APP minigene with the V717F mutation
(Note mutations are abbreviated as the amino acid at
location followed by the substituted amino acid). The

30   Tg2576 mouse [Hsiao et al, 1996] expresses the APP695
isoform containing a K670N,M671L mutation (APP770
numbering) which is often referred to as the Swedish
mutation. In addition to AD-type pathology, Tg2576
shows cognitive impairment as measured by spontaneous

35   alternation in a "Y" maze and spatial memory in a water

maze suggesting that the manipulation of APP affects
cognitive function in addition to pathology.

    However, other APP gene transgenic mice do not
produce or have weak FAD pathology. See Neve et al,
5   1996 for an example of weak pathology and Hsaio et al.,
1995; Andrä et al., 1996; Malherbe et al., 1996; Mucke
et al., 1994 for examples of APP mice which do not show
pathology and see Greenberg et al, 1996 for a review of
additional APP gene transgenic mice which do not show
10   pathology or show only weak pathology.

    Analysis of the above suggests that these
Alzheimer's models suffer from the limitation that they
are unable to produce sufficient amounts of $A\beta$ in the
brain to initiate Alzheimer's related pathology.
15   Therefore transgenic models producing sufficient
amounts of $A\beta$ in the brain in an accelerated manner and
methods of making such transgenics are needed.

    Recently, mutations in other genes, termed the
Presenilin I (PSI) and Presenilin II (PS2) genes
20   located on chromosomes 14 and 1 respectively, have also
been shown to cause FAD. [Cruts, et al., 1996].
Research has demonstrated that peripheral cells from
individuals with these presenilin mutations produce a
greater amount $A\beta1-42(43)$ than that produced in
25   individuals having a non-mutant PS gene [Scheuner et
al., 1996]. It has been suggested that the mode of
pathogenesis produced by these mutated presenilin genes
involves the production of more $A\beta1-42(43)$ relative to
the amount produced by a nonmutant (wildtype) PS1 or
30   PS2 gene. Presently, the mechanism which causes this
increased production of $A\beta1-42(43)$ is not known.

    Transgenic mice carrying mutations in PS1 do not
appear to develop AD-type pathology but do show an
elevation of $A\beta42(43)$ [see Example herein]. This form
35   of $A\beta$ is highly amyloidogenic and forms the early core
of amyloid deposits in AD brain [Mann et al, 1996;

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Jarrett et al, 1993]. Both PS1 and PS2 are known to
influence APP processing [Scheuner et al, 1996 and see
Example herein]. Sequence homology between the
presenilins and a *C. elegans* protein involved in
5   protein trafficking (SPE4) suggests that the
presenilins may direct the compartmentalization and
trafficking of APP [L'Hemault and Arduengo, 1992] and
that mutant presenilins may direct APP along a pathway
that results in elevated levels of $A\beta42(43)$. In this
10   way the biosynthetic pathways can be considered to be
interactive.

The above transgenic animals provide the current
models of Alzheimer's Disease. However, as discussed
herein above many of these models are incomplete, in
15   that the full pathology seen in humans is not seen, or
as seen in the PS models, no pathology just elevated
levels of $A\beta1-42(43)$ are seen. Further, the models
generally require that the mice age and the pathology
is not seen until nine months and generally later
20   [Hsaio et al., 1996]. This means that the animals must
be maintained for extended periods of time. The cost
of maintenance makes it difficult for many
investigators to use these models.

It would be useful therefore to have transgenic
25   models which show the full range of pathology of AD at
an earlier age or a selected aspect of the pathology or
for that matter any other human genetically based
condition. The method should allow the modulation of
the phenotype resulting from the expression of the
30   transgenes. It would further be useful to have a model
for AD in which the pathology onset is earlier
(accelerated). Specifically, a model in which amyloid
accumulation is enhanced and accelerated.

35

SUMMARY OF THE INVENTION AND ADVANTAGES

The present invention provides a method of
preparing a transgenic animal model with enhanced,
accelerated pathology for Alzheimer's Disease (AD) and
the transgenic animal made by the method. The method
includes producing an F_1 generation by crossing a first
and second transgenic parent each carrying a different
expressible transgene for differing aspects of the same
desired phenotype associated with AD pathology. The
offspring of the F_1 generation are then screened and
those which carry a transgene from each parental
transgenic animal resulting in an enhanced pathology
for Alzheimer's Disease are selected.

In a preferred embodiment the AD-associated
pathology is for amyloid accumulation. In an
embodiment a mutant presenilin transgene and a
transgene for a mutant amyloid precursor protein are
used. In a further embodiment the mutant presenilin
transgene is the PS1 M146L mutation and the mutant
amyloid precursor protein transgene is the Swedish
mutation (APP695 isoform containing a K670N,M671L
mutation (APP770 numbering)).

The present invention also provides a method of
modulating the phenotype of a transgenic animal model
and the transgenic organism with the modulated
phenotype. An F_1 generation is produced by crossing a
first and second transgenic parent, each carrying a
different expressible transgene at different steps in
the same and/or interactive biosynthetic pathway for
the desired phenotype of a transgenic animal. The
offspring are screened and offspring selected in the F_1
generation which carry a transgene from each transgenic
parent thereby providing a modulated phenotype.

The method of the present invention also provides
a method of screening for two expressible transgenes
which when expressed in the same animal result in a

modulated phenotype. In this method an F_1 generation is
produced by crossing a first and second transgenic
parent each carrying a different expressible transgene
at different steps in the same or an interactive
5   biosynthetic pathways for the phenotype under
consideration. Offspring are identified among the F_1
generation which carry a transgene from each transgenic
parent and which result in a modulated phenotype. When
offspring are identified with a modulated phenotype, a
10   transgenic animal carrying the two expressible
transgenes can be made.

DESCRIPTION OF THE DRAWINGS.

15   Other advantages of the present invention will be
readily appreciated as the same becomes better
understood by reference to the following detailed
description when considered in connection with the
accompanying drawings wherein:
20

FIGURE 1A-C are graphs showing early behavioral
changes in transgenic mice. Mice were tested for
spontaneous alternation behavior in a symmetrical "Y"
maze at 12-14 weeks. The alternation performance was
25   decreased in the parent carrying the APP695 isoform
containing a K670N,M671L mutation and doubly transgenic
mice compared to M146L and non-transgenic litter mates
(Fig. 1A). The activity of the mice in the "Y" maze
was elevated in the doubly transgenic mice relative to
30   all other genotypes (Fig. 1B). The frequency plot of
"Y" maze activity measurements for each mouse indicates
a right-shift in the frequency distribution (Fig. 1C).
Doubly transgenic mice (dashed line; n=21) and the
frequency distribution of the non-transgenic plus M146L
35   mice (solid line; n=63) which had identical means and
standard deviations (see Fig. 1B).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method of
producing a successor generation of transgenic animals
5   with a modulated phenotype from that of the
original/initial transgenic animals. The modulation
can be either an enhancement of the original observed
phenotype seen in the initial transgenic animals or a
diminution of the phenotype. By modulation is meant
10   that the characteristic phenotype shown by the
transgene is more pronounced, appears earlier or later;
where protein is produced more or less protein is
produced than the parent strains or the like. Where
earlier or accelerated, it is meant that the observed
15   phenotype is seen at least one month earlier in the
lifespan than the phenotype in the parental strain or
similarly for later appearance.

For example, a modulated phenotype for a human
disease model would show a pathology associated with
20   the disease that more accurately reflects the human
pathologic state including having more of the
characteristics of the disease than the initial
transgenic animal parental strains or the like.
Alternatively, a modulated phenotype could reflect a
25   faster or slower onset of the pathology of the human
disease.

Specifically, the method produces an F_1 generation
by crossing a first and second transgenic parent
specifically selected to complement each other. By
30   complement is meant that the transgenic parent carries
a different expressible transgene at different
steps/aspects in the same or interactive biosynthetic
pathway for the phenotype or phenotype aspect that is
to be modulated. In general the term aspect is used to
35   describe a significant part/facet/feature of the
phenotype that is being modulated. In general the term

biosynthetic pathway is used as is known in the art.
However, in producing a phenotype often the products of
one biosynthetic pathway must interact with those of
another to produce the observed phenotype and so the
term can be used in a broader concept to include
several specific biosynthetic pathways which interact
to provide an observed phenotype.

In general where the transgene carries a mutation
it is referred to by the mutation. The mutation is
abbreviated by the non-mutant amino acid followed by
the location in the sequence followed by the
substituted amino acid.

The transgene generally is a human gene where a
human condition is being studied. The transgene
(generally in the form of cDNA) can be the nonmutant
(often referred to as "wildtype") or a mutant human
gene. Additionally, a nonmutant transgene can also be
treated as is known in the art to express mutations.

The offspring of the F_1 generation with the
modulated phenotype are utilized in animal models as
for example testing of treatment modalities in a
disease model or for pathogen susceptibility.

Further, one parent strain instead of carrying a
direct human transgene may have the homologous
endogenous gene modified by gene targeting such that it
approximates the transgene. That is, the endogenous
gene has been "humanized" and/or mutated [Reaume et al,
1996]. It should be noted that if the animal and human
sequence are essentially homologous a "humanized" gene
is not required. The transgenic parent can also carry
an overexpressed sequence, either the nonmutant or a
mutant sequence and humanized or not as required. The
term transgene is therefore used to refer to all these
possibilities.

Additionally, cells can be isolated from the
offspring which carry a transgene from each transgenic

parent and that are used to establish primary cell
cultures or cell lines as is known in the art.

Where appropriate, a parent strain will be
homozygous for the transgene. Additionally, where
5   appropriate, the endogenous nontransgene in the genome
that is homologous to the transgene will be
nonexpressive. By nonexpressive is meant that the
endogenous gene will not be expressed and that this
nonexpression is heritable in the offspring. For
10   example, the endogenous homologous gene could be
"knocked-out" by methods known in the art.
Alternatively, the parental strain that receives one of
the transgenes could carry a mutation at the endogenous
homologous gene rendering it nonexpressed.
15   The method of the present invention also provides
a screening protocol to determine which combination of
transgenes produce modulated phenotypes. These
combinations can then be incorporated in a single
transgenic animal to produce a modulated phenotype.
20   That is, an engineered transgenic animal may be wanted
that has incorporated in its genome two or more
transgenes but that is not established by crossing
rather is engineered by standard methods for making
transgenic animals. For example, a transgenic organsim
25   may be wanted wherein two or more transgenes are on the
same vector under the control of the same promoter and
which should have the required modulated phenotype.
However due to the cost and effort of making the
transgenic animal a screening is necessary to determine
30   the most likely candidates to engineer.

In the screening method of the present invention
an F_1 generation is produced by crossing a first and
second transgenic parent each carrying a different
expressible transgene at different steps/aspects in the
35   same or interactive biosynthetic pathways for the
phenotype or phenotype aspect under consideration. The

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offspring of the F$_1$ generation are tested and those
offspring which carry a transgene from each transgenic
parent are selected and observed for the modulated
phenotype.

5   The transgenic parents are produced as is known in
the art. The present invention provides for transgenic
parental strains containing transgenes as described
herein above and including gene targeted or
overexpressed mutant or nonmutant transgenes and where
10   appropriate as well as for knockout strains carrying a
transgene. Any method can be used which provides for
stable, inheritable, expressible incorporation of the
transgene within the nuclear DNA of an animal. These
transgenic animals are constructed using standard
15   methods known in the art and as set forth in United
States Patents 5,614,396 5,487,992, 5,464,764,
5,387,742, 5,347,075, 5,298,422, 5,288,846, 5,221,778,
5,175,384, 5,175,383, 4,873,191, 4,736,866 as well as
Burke and Olson [1991], Capecchi [1989], Davies et al.
20   [1992], Dickinson et al. [1993], Duff and Lincoln
[1995], Huxley et al. [1991], Jakobovits et al. [1993],
Lamb et al. [1993], Pearson and Choi [1993], Rothstein
[1991], Schedl et al. [1993], Strauss et al. [1993].
Further, patent applications WO 94/23049, WO 93/14200,
25   WO 94/06908, WO 94/28123 also provide information.

More specifically, any techniques known in the art
may be used to introduce the transgene expressibly into
animals to produce the parental lines of animals. Such
techniques include, but are not limited to, pronuclear
30   microinjection (U.S. patent 4,873,191); retrovirus
mediated gene transfer into germ lines [Van der Putten
et al., 1985]; gene targeting in embryonic stem cells
[Thompson et al., 1989 and U.S. patent 5,614,396];
electroporation of embryos [Lo, 1983]; and sperm-
35   mediated gene transfer [Lavitrano et al., 1989]. For a
review of such techniques see Gordon [1989].

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        In an exemplar of the present invention as shown
    in the Example herein below a transgenic animal has
    been created for Alzheimer's Disease (AD) having a
    pronounced accelerated phenotype of an Aβ related
5   phenotype of accumulated amyloid using the method of
    the present invention. The animal is a rodent and in a
    preferred embodiment, a mouse.
        As discussed herein above the pathology of AD is
    related to aberrant amyloid production. Therefore in
10 this transgenic model, genes relating to the
    biosynthetic pathways for the phenotype of amyloid
    accumulation have been used.
        In a preferred embodiment for a model for AD the
    first parental animal carries an expressible transgene
15 for a mutant presenilin transgene [Cruts et al., 1996;
    Duff et al., 1996] and the second parental animal
    carries an expressible transgene for a mutant amyloid
    precursor protein [U.S. patents 5,612,486; 5,602,299;
    5,525,714; 5,604,131; 5,387,742; 5,151,508; PCT Patent
20 Applications WO 93/14200; WO 96/06927; WO 94/12627; WO
    93/02189; WO 92/06187; European Patent Applications EP
    0 451 700 A1; EP 0 653 154 A2; Neve et al., 1996; Hsiao
    et al., 1996; Ali et al., 1996]. The APP gene can be a
    humanized targeted nonmutant sequence or mutant
25 sequence and these can be overexpressed or not.
    Further overexpressed animal nonmutant or mutant
    sequences can also be used. The presenilin genes (PS1
    and PS2) in the mouse and human have essentially
    homologous sequences so that targeted mutations can be
30 used to generate the parent strain without the need to
    humanize first.
        More specifically, the mutant presenilin transgene
    is M146L and the mutant amyloid precursor protein (APP)
    transgene is for an APP695 isoform containing a
35 K670N,M671L mutation. However, any mutant presenilin
    or amyloid precursor protein gene or subsequently

discovered homologs can be used that provide
complementarity/interactivity for the expressed
amyloid-associated AD phenotype of accumulation Aβ. In
further embodiments overexpressed C terminal fragments

5   of APP is used, or the PDAPP mouse [Games et al, 1995],
and other APP mice [Sommer et al, 1996; Lamb et al,
1993; Reaume et al., 1996] can be used.

Utilizing the method of the present invention, F_1
progeny from a cross between a parental mouse carrying

10 the mutant APP transgene for an APP695 isoform
containing a K670N,M671L mutation (referred to herein
as K670N,M671L) and a parental mouse carrying the
mutant PS1 transgene for M146L were bred and examined
in relation to histopathology and behavioral phenotype.

15 In these animals, the expression of mutant PS1 in
addition to the expression of mutant APP, accelerates
the rate at which Aβ is deposited, most likely due to
the PS1-mutant driven elevation in Aβ1-42(43).   The
double transgenic mice had accelerated formation of

20 deposits containing Aβ (Table 1). Sections stained
with the antibody 4G8 (which stains both human and
mouse Aβ ending at either residue 40 or residue 42)
labels numerous deposits in every doubly transgenic
mouse killed between 26 and 32 weeks of age, generally

25 at 29 weeks of age. Corresponding areas from
littermate age-matched single transgenic K670N,M671L or
M146L mice and non-transgenic littermates were negative
for Aβ deposits.   The positively staining Aβ deposits
become apparent by 13 weeks of age and increased in

30 number and size from that time point showing a
modulated phenotype which accelerates the process of Aβ
accumulation in the doubly transgenic mice.

All mice in these studies were subjected to a
behavioral test battery at 12-14 weeks old. All groups

35 of mice performed similarly on test of sensory and
motor function. Body weights were similar for all

genotypes. However, when mice were tested for
spontaneous alternation behavior in the symmetrical "Y"
maze, differences were observed in the total number of
arm entries. This index of activity was modified by

5   genotype. The doubly transgenic mice had increased
activity compared with the K670N,M671L or M146L or non-
transgenic littermates. Thus, as early as 12 weeks,
there is a synergistic effect of PS1 and APP mutations
on this behavioral measure.

10   These results clearly demonstrate that presenilin
mutations accelerate development of the AD phenotype in
K670N,M671L mice in a synergistic manner and provides a
modulated phenotype. The data indicate that mutant
presenilin affects the processing of mutant APP

15   expressed from the K670N,M671L transgene to enhance Aβ
production, fibrillar plaque formation and affect
behavior early in the life-spans of mice. The rapid
development of the AD phenotype in these mice will be
advantageous in addressing mechanistic issues of

20   amyloid toxicity, and testing the efficacy of agents
proposed to interact with select aspects of the AD
phenotype.

The above discussion provides a factual basis for
the method of developing transgenic animals which have

25   a modified phenotype. The methods used with and the
utility of the present invention can be shown by the
following non-limiting examples and accompanying
figures.

30   **EXAMPLES**

GENERAL METHODS:

General methods in immunohistochemistry: Standard
methods known in the art and not specifically described
were generally followed as in Stites et al.(eds), Basic

35   and Clinical Immunology (8th Edition), Appleton &
Lange, Norwalk, CT (1994) and Johnstone & Thorpe,

Immunochemistry in Practice, Blackwell Scientific
Publications, Oxford, 1982.

General methods in molecular biology: Standard
molecular biology techniques known in the art and not
specifically described were generally followed as in
Sambrook et al., *Molecular Cloning: A Laboratory
Manual*, Cold Springs Harbor Laboratory, New York (1989,
1992), and in Ausubel et al., *Current Protocols in
Molecular Biology*, John Wiley and Sons, Baltimore,
Maryland (1989).

Genotyping Litters:

The resulting offspring were genotyped by cutting
tail tips from weanlings, extracting DNA using a
Promega genomic DNA extraction kit, transferring
denatured DNA to nylon membrane and hybridizing
duplicate samples with a DNA probe that recognizes
either APP or PS1.

Sensory and Motor Function Tests: For all
litters, mice were weighed between 12 and 14 weeks.
The forelimb placing response of each mouse was checked
by holding the mouse by the tail above a flat surface
and slowly lowering the mouse to see if the forepaws
reached outwards towards the surface. The righting
reflex displayed when the mice were held with feet
upwards and dropped from a height of 35 cm onto a soft
surface of bedding, was used to test vestibular
function. A loud metallic click to elicit the Preyer
reflex (a backwards flick of the pinna) was used to
test hearing. Testing for spontaneous seizures was
accomplished by stressing each mouse by holding it by
the tail for 30 seconds. Mice were then placed back in
a cage and observed after a 30 minute interval for
seizure activity. Motor coordination was investigated
using the coat hanger test as described by Lalonde and
Thifault (1994).

Behavioral Tests: Three month old mice were also
tested for spontaneous alteration, using a protocol
similar to those described by Hsiao [1996] and Maurice
et al. [1996]. Mice were placed in the center of a
5   symmetrical "Y" maze made of white acrylic and the
sequence of arm entries over 8 minutes were recorded.
Percentage alternation is the number of triads
containing entries into all three arms (i.e. ABC, ACB,
CAB, etc.) divided by maximum possible alternations
10   (equivalent to the total number of arms entered minus
2) x 100.

For statistical analysis, littermates of the same
genotype were averaged, and the values for each litter
were compared by ANOVA followed by Newman-Keuls to
15   determine differences between individual groups (n =
11-12 litters for each genotype; 33 non-transgenic, 30
M146L, 22 K670N,M671L and 20 doubly transgenic mice).

Immunohistochemical Protocols: Doubly transgenic
(n=6), single transgenic K670N,M671L (n=5) and M146L
20   (n=3) and non-transgenic mice (n=4; all 26-32 weeks
old), and one 15 month old parental K670N,M671L mouse
were sacrificed. One hemisphere of the brain was
gently shaken in 0.9% saline for 30 minutes and
immersion fixed in 4% paraformaldehyde for 24 hours,
25   cryoprotected in 15% sucrose/PBS (2 hours) followed by
an overnight immersion in 30% sucrose/PBS. Twenty five
μm horizontal sections were prepared with a sliding
microtome.

Immunohistochemistry was performed on floating
30   sections using standard protocols. Primary antibodies
recognizing Aβ residues 17-24 (4G8, Senetek, Maryland
Heights, MO, 1:1000) and GFAP (Boehringer Mannheim,
Indianapolis, IN, 1:1000) were used. GFAP stained
sections were mounted on slides and counterstained with
35   Congo red (Sigma Accustain Amyloid staining kit, St.
Louis, MO). Congo red staining was evaluated using

cross-polarized illumination. Only deposits that were
green/red birefringement when the polarizer was rotated
were counted as Congo red positive deposits.
Additional sections were stained with 1% thioflavin S
5   after 10 minutes in Mayer's hematoxylin to mask nuclear
fluorescence. Counts of plaques were all made at 40x
total magnification at each of 3 different dorsoventral
planes.

10   RESULTS
        Progeny from a cross between parental mice
carrying the APP695 isoform containing a K670N,M671L
mutation (APP770 numbering) and parental mice carrying
a mutant PS1 transgene M146L were examined in relation
15   to histopathology and behavioral phenotype. In these
animals, the expression of mutant PS1 in addition to
the expression of mutant APP, accelerates the rate at
which Aβ is deposited, most likely due to the PS1-
mutant driven elevation in Aβ1-42(43). All animals
20   expressing the *APP* transgene show deficits in the
behavioral test, spontaneous alternation, but this does
not correlate with the deposition of Aβ suggesting that
these deficits of cognitive impairment are not entirely
related to a toxic component of visible amyloid
25   deposits.
        First litter progeny (19 animals) from the cross
were sacrificed between 26 and 32 weeks of age. The
histopathology from these animals shows that the double
transgenic mice had accelerated formation of deposits
30   containing Aβ relative to singly transgenic
littermates. Sections stained with the antibody 4G8
(which stains both human and mouse Aβ ending at either
residue 40 or residue 42) labels numerous deposits in
every doubly transgenic mouse sacrificed between 26 and
35   32 weeks of age (6 mice, all three sections examined in
each case). These deposits are also stained by Congo

red when viewed under polarized light. Corresponding
areas from age-matched K670N,M671L and M146L
littermates and non-transgenic littermates were
negative for Aβ deposits (see Table 1).

5   Thioflavin S positive deposits were only found in
the double mutant mice cortex and hippocampus, with a
slightly lower density in the more ventral regions of
cortex. No deposits were observed in any other brain
regions. Some of the deposits resemble a Maltese cross

10   when stained with Congo red and viewed under cross-
polarized light. Similar structures are seen in human
AD brains. The number of thioflavin S deposits
consistently exceeded the numbers found with Congo red
or Aβ immunohistochemistry, but the values were highly

15   correlated within the double mutant mice (r=0.93 for
thioflavin S and Congo red and r=0.81 for thioflavin S
and Aβ immunohistochemistry). These high correlation's
suggest all three measurements reflect the same process
of deposition, which varies slightly in each mouse.

20   The higher absolute values for thioflavin S indicates
the deposits are easier to identify with this
technique.

The positive reaction of all three methods with
the deposits in the doubly transgenic mice strongly

25   indicates that they consist of fibrillar Aβ amyloid.
As a control, a 15 month old parental K670N,M671L mouse
was sacrificed and processed in parallel with the 26-32
week old mice. This mouse had deposits which were
positively immunostained with Aβ, Congo red and

30   thioflavin S.

Reactive astrocytes are seen surrounding the
deposits when the slides are stained with a monoclonal
antibody to glial fibrillary acidic protein (GFAP).
The astrocytes appear in clusters within the cerebral

35   cortex of the doubly transgenic mice. Few if any
clusters of reactive astrocytes are present in 26-32

week old K670N,M671L mice, M146L littermates or non-transgenic littermates. Double-staining with Congo red revealed amyloid deposits in the center of these clusters of reactive astrocytes. Astrocytes clusters were present also in the hippocampus, but were not as discernible as in neocortex because of the higher basal GFAP content in this region of mouse brain. Similar clusters were apparent in the 15 month old parental K670N,M671L mouse.

Four younger doubly transgenic mice were sacrificed at 13 (n=2) or 15 (n=2) weeks of age. The 13 week old mice did not have Aβ deposits evaluated either by Aβ immunochemistry or Congo red staining. The 15 week old mice averaged one deposit per section, however, thioflavin S stained deposits were visible in both 13 and 15 week old doubly transgenic mice. Some of the thioflavin S deposits consisted of only a few thread-like profiles. The deposits were very small, and substantially fewer in number (less than 5 per section) than those in the 26-32 week old mice (average of 77 per section; Table 1). Thus, the Aβ deposits are becoming apparent by 13 weeks of age and increase in number and size from that time.

All mice in these studies were subjected to a sensory and motor function and behavioral test battery at 12-14 weeks old. All groups of mice performed similarly on tests of sensory and motor function including a wire hang test (strength and coordination), righting response (vestibular), Preyer's response (auditory) and the forelimb placing response (visual and motor). Body weights were similar for all genotypes.

However, when mice were tested for spontaneous alternation behavior in the symmetrical "Y" maze, differences were observed. Alternation performance was modified by genotype (Fig. 1A; ANOVA: $F(3,43)=5.64$,

p<0.005). The percentage of alternations was
significantly lower in both the doubly transgenic and
K670N,M671L mice compared to the M146L or non-
transgenic littermates (p<0.05; Neman-Keul's post-hoc
5   comparisons). These results indicate that mice
expressing an APP mutation have impaired alternation
performance at a very early age. This behavioral
deficit may be caused by overexpression of mutant APP,
or elevated levels of soluble human Aβ. Importantly,
10   this deficit is not caused by formation of visible
fibrillar Aβ deposits because these are either absent
or rare in 12 weeks old doubly mutant mice and even by
32 weeks, these deposits are not seen in K670N,M671L
mice.

15   A second measurement in the "Y" maze alternation
test is the total number of arm entries. This index of
activity was also affected by genotype (Fig. 1B; ANOVA:
$F(3,43)=3.73$, p<0.02). The doubly transgenic mice had
increased activity compared with the single transgenic
20   K670N,M671L, M146L or non-transgenic mice (p<0.05;
Newman-Koul's post-hoc comparisons). The K670N,M671L
mice were not significantly different from the other
two genotypes on this measure. Figure 1C demonstrates
the rightward shift of the frequency distribution for
25   the activity scores of doubly transgenic mice,
indicating this is not the result of the a new outlying
animals. Thus, as early as 12 weeks, there is a
synergistic effect of PS1 and APP mutations on this
behavioral measure.

30   These results clearly demonstrate that presenilin
mutations accelerate development of the AD phenotype in
K670N,M671L mice in a synergistic manner. The data
indicate that mutant presenilin affects the processing
of mutant APP expressed from the K670N,M671L transgene
35 . to enhance fibrillar plaque formation and affect
behavior early in the life-spans of mice. The rapid

development of the AD phenotype in these mice will be
advantageous in addressing mechanistic issues of
amyloid toxicity, and, potentially, testing the
efficacy of agents proposed to interact with select
5   aspects of the AD phenotype.

Throughout this application, various
publications, including United States and other
patents, are referenced by author and year and patents
by number. Full citations for the publications are
10   listed below. The disclosures of these publications
and patents in their entireties are hereby incorporated
by reference into this application in order to more
fully describe the state of the art to which this
invention pertains.

15   The invention has been described in an
illustrative manner, and it is to be understood that
the terminology which has been used is intended to be
in the nature of words of description rather than of
limitation.

20   Obviously, many modifications and variations of
the present invention are possible in light of the
above teachings. It is, therefore, to be understood
that within the scope of the appended claims, the
invention may be practiced otherwise than as
25   specifically described.

TABLE 1

DEPOSIT NUMBER IN DOUBLY

TRANSGENIC AND K670N,M671L MICE

Doubly Transgenic Mice
plaque counts/section

Mouse	Age	TS	CR	Aβ (IHC)
F2	26	77	34	58
F7	26	61	26	31
G3	26	48	24	34
G6	26	97	43	53
C7	29	78	42	48
A5	32	101	44	54

K670N,M671L Mice
plaque counts/section

Mouse	Age	TS	CR	Aβ (IHC)
H6	24	0	0	0
F5	26	0	0	0
E1	27	0	0	0
D3	28	0	0	0
C1	29	0	0	0

Mice ages (weeks) with plaque numbers per
section counted from thioflavin S (TS), Congo
Red (CR) or Aβ immunohistochemistry (IHC).

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CLAIMS

What is claimed is:

1.   A method of preparing a transgenic animal
with enhanced amyloid pathology for Alzheimer's Disease
by
        producing an F_1 generation by crossing a first
and second transgenic heterozygous parent each carrying
appropriately integrated into its genome a different
expressible transgene for different aspects of amyloid
accumulation and
        selecting among the offspring of the F_1
generation for those which carry a transgene from each
parental transgenic animal resulting in an enhanced
amyloid pathology for Alzheimer's Disease.

2.   The method as set forth in claim 1
wherein at least one transgenic parent is homozygous
for the transgene.

3.   The method as set forth in claim 1
wherein at least one transgenic parent has the
endogenous nontransgene homolog nonexpressive.

4.   The method as set forth in claim 1
wherein the first parental animal carries a transgene
for a mutant presenilin transgene and the second
parental animal carries a transgene for an amyloid
precursor protein.

5.   The method as set forth in claim 4
wherein the mutant presenilin transgene is M146L and
the amyloid precursor protein (*APP*) transgene is for an
APP695 isoform containing a K670N,M671L mutation.

6.   The method as set forth in claim 4
wherein the transgene for amyloid precursor protein is
a targeted humanized amyloid precursor protein gene
sequence that is mutant or nonmutant or is an
overexpressed sequence that is mutant or nonmutant and
which may be humanized.

7.   The method as set forth in claim 1
wherein the transgenic animal is a rodent.

8.   The method as set forth in claim 7
wherein the rodent is a mouse.

9.   A transgenic animal which exhibits an
enhanced selected pathology for Alzheimer's Disease
produced by
          producing an F_1 generation by crossing a first
heterozygous parental animal containing appropriately
integrated into its genome an expressible transgene for
a selected pathology for Alzheimer's Disease and a
second heterozygous parental animal containing
integrated into its genome an expressible transgene for
the same selected pathology for Alzheimer's Disease;
and
          selecting among the F_1 generation for those
which carry both expressible transgenes resulting in an
enhanced accelerated selected pathology for Alzheimer's
Disease than that for the parental strains.

10.   The transgenic animal as set forth in
claim 9 wherein at least one transgenic parent is
homozygous for the transgene.

11.   The transgenic animal as set forth in
claim 9 wherein at least one transgenic parent has the
endogenous nontransgene homolog nonexpressive.

12.   The transgenic animal as set forth in claim 9 wherein the transgenic animal is a rodent.

13.   The transgenic animal as set forth in claim 12 wherein the rodent is a mouse.

14.   The transgenic animal as set forth in claim 9 wherein the selected pathology for Alzheimer's Disease is amyloid accumulation.

15.   The transgenic animal as set forth in claim 9 wherein the selected pathology for Alzheimer's Disease is intraneuronal tangles.

16.   The transgenic animal as set forth in claim 14 wherein the first parental animal carries a transgene for a mutant presenilin transgene and the second parental animal carries a transgene for an amyloid precursor protein.

17.   The transgenic animal as set forth in claim 16 wherein the mutant presenilin transgene is M146L and the amyloid precursor protein transgene is for an APP695 isoform containing a K670N,M671L mutation.

18.   A transgenic mouse which exhibits enhanced, accelerated pathology for Alzheimer's Disease within six months of birth containing appropriately integrated into its genome an expressible transgene for amyloid precursor protein APP695 isoform containing a K670N,M671L mutation and a second expressible transgene for mutant presenilin M146L.

19.   The transgenic mouse as set forth in claim 18 wherein at least one transgene is homozygous.

20.   The transgenic mouse as set forth in
claim 18 wherein at least one endogenous nontransgene
homolog is nonexpressive.

21.   A method of modulating the phenotype of
a transgenic animal by
        producing an F_1 generation by crossing a first
and second heterozygous transgenic parent each carrying
a different expressible transgene for different aspects
in the same or interactive biosynthetic pathways for
expression of the phenotype of a transgenic animal; and
        selecting among the offspring of the F_1
generation for those which carry a transgene from each
transgenic parent resulting in a modulated phenotype.

22.   The method as set forth in claim 21
wherein at least one transgenic parent is homozygous
for the transgene.

23.   The method as set forth in claim 21
wherein at least one transgenic parent has the
endogenous nontransgene homolog nonexpressive.

24.   The method as set forth in claim 21
wherein the modulated phenotype is for an Alzheimer's
Disease phenotype.

25.   The method as set forth in claim 24
wherein the pathways are for the accumulation of
amyloid.

26.   The method as set forth in claim 25
wherein the first parental animal carries a transgene
for a mutant presenilin transgene and the second
parental animal carries a transgene for an amyloid
precursor protein.

27.   The method as set forth in claim 26
wherein the mutant presenilin transgene is M146L and
the amyloid precursor protein transgene is for an
APP695 isoform containing a K670N,M671L mutation

28.   A method of screening for two
expressible transgenes which when expressed in the same
animal result in a modulated phenotype by
    producing an F_1 generation by crossing a first
and second heterozygous transgenic parent each carrying
a different expressible transgene at different steps in
the same or compatible biosynthetic pathway for
expressing the phenotype;
    identifying offspring among the F_1 generation
which carry a transgene from each transgenic parent and
which result in a modulated phenotype.

1/1

Fig-1A

Fig-1B

Fig-1C

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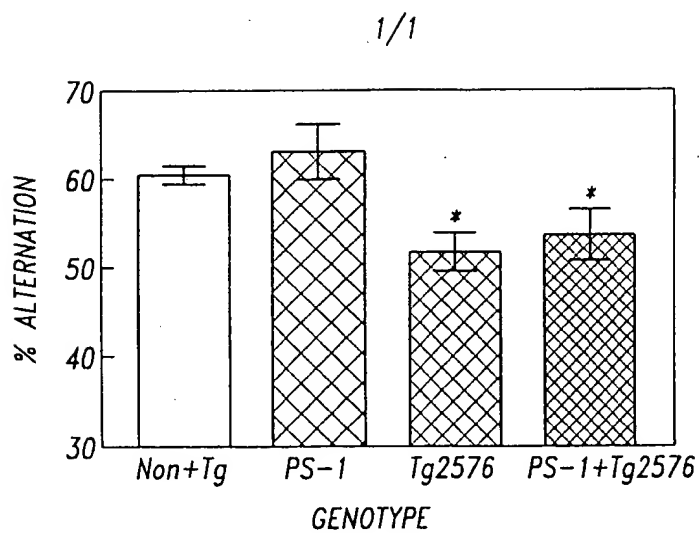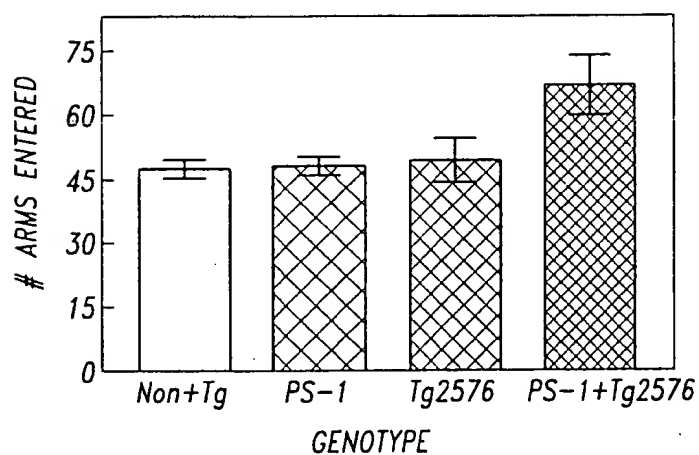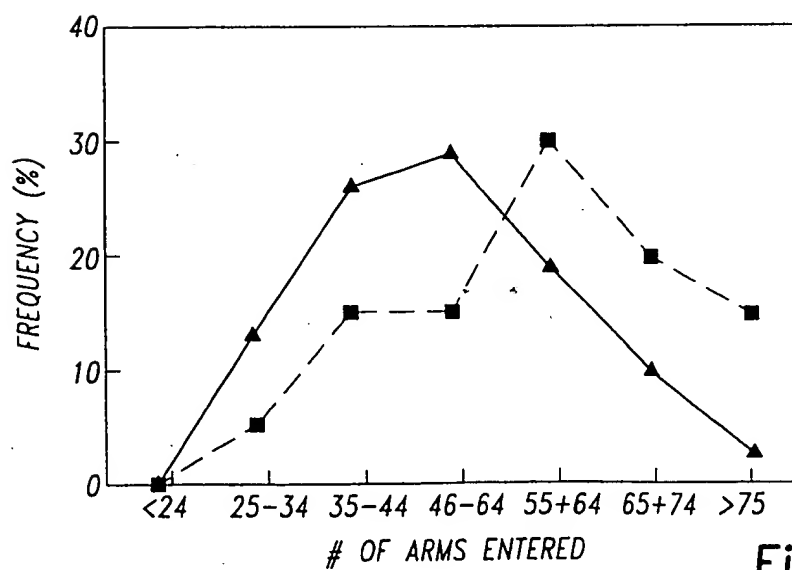
INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C12N 5/00, 15/00; A61K 49/00
US CL   :435/172.3; 800/2; 424/9.2

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

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U.S. : 435/172.3; 800/2; 424/9.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS, STN

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/11968 A1 (ATHENA NEUROSCIENCES, INC.) 04 May 1995, pages 1-48, especially pages 41-47	1-28
Y	LEVY-LAHAD, E. Candidate Gene for the Chromosome 1 Familial Alzheimer's Disease Locus. Science. 18 August 1995, Vol. 269, pages 973-977, especially page 975.	1-28
Y	ROGAEV, E.I. et al. Familial Alzheimer's Disease in Kindreds with Missense Mutations in a Gene on Chromosome 1 Related to the Alzheimer's Disease Type 3 Gene. Nature. 31 August 1995, Vol. 376, page 775-778, especially page 776.	1-28

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

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(54) Title: METHOD FOR PRODUCING TRANSGENIC ANIMAL MODELS WITH MODULATED PHENOTYPE AND ANIMALS PRODUCED THEREFROM

(57) Abstract

A method of preparing a transgenic animal model with enhanced, accelerated pathology for Alzheimer's Disease (AD) and the transgenic animal made by the method are disclosed. The method includes producing an F_1 generation by crossing a first and second transgenic parent each carrying a different expressible transgene for differing aspects of the same desired phenotype associated with AD pathology. The offsprings of the F_1 generation are then screened and those which carry a transgene from each parental transgenic animal resulting in an enhanced pathology for Alzheimer's Disease are selected. In a preferred embodiment the AD–associated pathology is for amyloid accumulation. In an embodiment a mutant presenilin transgene and a transgene for a mutant amyloid precursor protein are used. In a further embodiment the mutant presenilin transgene is the PS1 M146L mutation and the mutant amyloid precursor protein transgene is the Swedish mutation (app695 isoform containing a K670N, M671L mutation (app770 numbering)).